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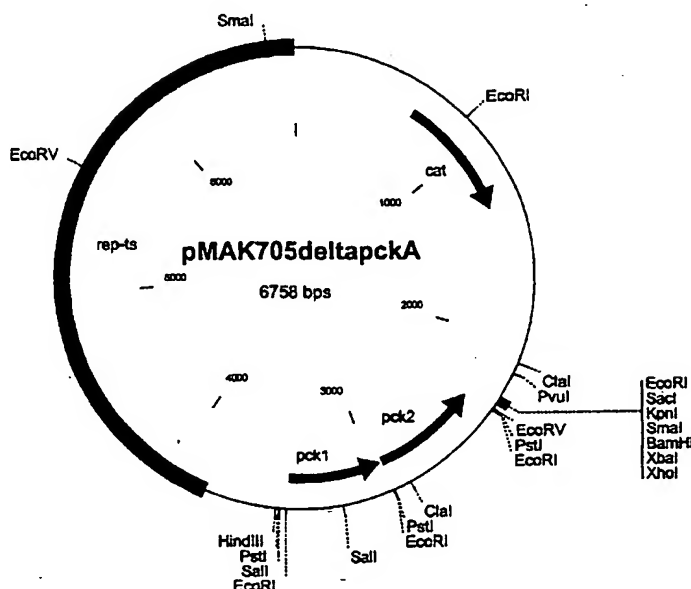
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(54) Title: FERMENTATION PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE



(57) Abstract: The invention relates to a fermentation process for the preparation of L-amino acids, especially L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which microorganisms at least the pckA gene and/or the open reading frames yjfa and ytfP are, individually or jointly, attenuated and, in particular, switched off, b) enrichment of the L-amino acid in the medium or in the bacterial cells, and c) isolation of the L-amino acid.



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**Fermentation Process for the Preparation of L-Amino Acids
using Strains of the Family Enterobacteriaceae**

Field of the Invention

The present invention relates to a fermentation process for
5 the preparation of L-amino acids, especially L-threonine,
using strains of the family Enterobacteriaceae in which at
least the pckA gene is attenuated.

Prior Art

L-Amino acids are used in animal nutrition, in human
10 medicine and in the pharmaceutical industry.

It is known to prepare L-amino acids by the fermentation of
strains of Enterobacteriaceae, especially Escherichia coli
and Serratia marcescens. Because of their great
importance, attempts are constantly being made to improve
15 the preparative processes. Improvements to the processes
may relate to measures involving the fermentation
technology, e.g. stirring and oxygen supply, or the
composition of the nutrient media, e.g. the sugar
concentration during fermentation, or the work-up to the
20 product form, e.g. by ion exchange chromatography, or the
intrinsic productivity characteristics of the microorganism
itself.

The productivity characteristics of these microorganisms
are improved by using methods of mutagenesis, selection and
25 mutant choice to give strains which are resistant to
antimetabolites, e.g. the threonine analogue α -amino- β -
hydroxyvaleric acid (AHV) or auxotrophic for metabolites of
regulatory significance, and produce L-amino acids, e.g. L-
threonine.

30 Methods of recombinant DNA technology have also been used
for some years to improve L-amino acid-producing strains of

the family Enterobacteriaceae by amplifying individual amino acid biosynthesis genes and studying the effect on production.

Object of the Invention

- 5 The object which the inventors set themselves was to provide novel procedures for improving the preparation of L-amino acids, especially L-threonine, by fermentation.

Summary of the Invention

- The invention provides a fermentation process for the
10 preparation of L-amino acids, especially L-threonine, using microorganisms of the family Enterobacteriaceae which, in particular, already produce L-threonine and in which the nucleotide sequence (pckA gene) coding for the enzyme phosphoenolpyruvate carboxykinase (PEP carboxykinase)
15 (EC 4.1.1.49) is attenuated.

Detailed Description of the Invention

- Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-
20 asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-homoserine and L-arginine. L-Threonine is particularly preferred.

- 25 In this context the term "attenuation" describes the reduction or switching-off, in a microorganism, of the intracellular activity of one or more enzymes (proteins) which are coded for by the appropriate DNA, for example by using a weak promoter or a gene or allele which codes for
30 an appropriate enzyme with low activity, or inactivating

the appropriate enzyme (protein), and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

The process is characterized in that the following steps are carried out:

- a) fermentation of microorganisms of the family Enterobacteriaceae in which at least the pckA gene is attenuated,
- b) enrichment of the appropriate L-amino acid in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and
- c) isolation of the desired L-amino acid.

The microorganisms provided by the present invention can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, optionally starch or optionally cellulose, or from glycerol and ethanol. Said microorganisms are representatives of the family Enterobacteriaceae selected from the genera Escherichia, Erwinia, Providencia and Serratia. The genera Escherichia and Serratia are preferred. The species Escherichia coli and Serratia marcescens may be mentioned in particular among the genera Escherichia and Serratia respectively.

Examples of suitable strains, particularly L-threonine-producing strains, of the genus Escherichia, especially of the species Escherichia coli, are:

- Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935
Escherichia coli VNIIGenetika MG442
5 Escherichia coli VNIIGenetika M1
Escherichia coli VNIIGenetika 472T23
Escherichia coli BKIIM B-3996
Escherichia coli kat 13
Escherichia coli KCCM-10132.
- 10 Examples of suitable L-threonine-producing strains of the
genus Serratia, especially of the species Serratia
marcescens, are:
- Serratia marcescens HNr21
Serratia marcescens TLr156
15 Serratia marcescens T2000.

- L-Threonine-producing strains of the family
Enterobacteriaceae preferably possess, inter alia, one or
more genetic or phenotypic characteristics selected from
the group comprising resistance to α -amino- β -hydroxyvaleric
20 acid, resistance to thialysine, resistance to ethionine,
resistance to α -methylserine, resistance to diaminosuccinic
acid, resistance to α -aminobutyric acid, resistance to
borrelidine, resistance to rifampicin, resistance to valine
analogues such as valine hydroxamate, resistance to purine
25 analogues such as 6-dimethylaminopurine, need for L-
methionine, optionally partial and compensable need for L-
isoleucine, need for meso-diaminopimelic acid, auxotrophy
in respect of threonine-containing dipeptides, resistance
to L-threonine, resistance to L-homoserine, resistance to
30 L-lysine, resistance to L-methionine, resistance to L-
glutamic acid, resistance to L-aspartate, resistance to L-
leucine, resistance to L-phenylalanine, resistance to L-
serine, resistance to L-cysteine, resistance to L-valine,
sensitivity to fluoropyruvate, defective threonine

dehydrogenase, optionally capability for sucrose utilization, amplification of the threonine operon, amplification of homoserine dehydrogenase I-aspartate kinase I, preferably of the feedback-resistant form, 5 amplification of homoserine kinase, amplification of threonine synthase, amplification of aspartate kinase, optionally of the feedback-resistant form, amplification of aspartate semialdehyde dehydrogenase, amplification of phosphoenolpyruvate carboxylase, optionally of the 10 feedback-resistant form, amplification of phosphoenolpyruvate synthase, amplification of transhydrogenase, amplification of the RhtB gene product, amplification of the RhtC gene product, amplification of the YfiK gene product, amplification of malate quinone 15 oxidoreductase and amplification of a pyruvate carboxylase and attenuation of acetic acid formation.

It has been found that the production of L-amino acids, especially L-threonine, by microorganisms of the family Enterobacteriaceae is improved after attenuation and, in 20 particular, switching-off of the pckA gene coding for PEP carboxykinase (EC 4.1.1.49).

The nucleotide sequence of the pckA gene of Escherichia coli has been published by Medina et al. (Journal of Bacteriology 172, 7151-7156 (1990)) and can also be taken 25 from the genome sequence of Escherichia coli published by Blattner et al. (Science 277, 1453-1462 (1997)). The nucleotide sequence of the pckA gene of Escherichia coli is represented in SEQ ID No. 1 and the amino acid sequence of the corresponding gene product is represented in SEQ ID No. 30 2.

The pckA genes described in the above literature references can be used according to the invention. It is also possible to use alleles of the pckA gene which result from

the degeneracy of the genetic code or from neutral sense mutations.

Attenuation can be achieved for example by reducing or switching off the expression of the pckA gene or the
5 catalytic properties of the enzyme protein. Both measures may optionally be combined.

Gene expression can be reduced by an appropriate culture technique, by genetic modification (mutation) of the signal structures of gene expression, or by means of antisense RNA
10 technology. Examples of signal structures of gene expression are repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. Those skilled in the art will find relevant information inter alia in e.g. Jensen and Hammer
15 (Biotechnology and Bioengineering 58, 191-195 (1998)), Carrier and Keasling (Biotechnology Progress 15, 58-64 (1999)), Franch and Gerdes (Current Opinion in Microbiology 3, 159-164 (2000)) and well-known textbooks on genetics and molecular biology, for example the textbook by Knippers
20 ("Molekulare Genetik" ("Molecular Genetics"), 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or the textbook by Winnacker ("Gene und Klone" ("From Genes to Clones"), VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

25 Mutations which cause a change or reduction in the catalytic properties of enzyme proteins are known from the state of the art. Examples which may be mentioned are the studies of Qiu and Goodman (Journal of Biological Chemistry 272, 8611-8617 (1997)), Yano et al. (Proceedings of the
30 National Academy of Sciences USA 95, 5511-5515 (1998)) and Wenté and Schachmann (Journal of Biological Chemistry 266, 20833-20839 (1991)). Surveys can be found in well-known textbooks on genetics and molecular biology, e.g. the

textbook by Hagemann ("Allgemeine Genetik" ("General Genetics"), Gustav Fischer Verlag, Stuttgart, 1986).

Mutations to be taken into consideration are transitions, transversions, insertions and deletions. Depending on the effect of amino acid exchange on the enzyme activity, the term missense mutations or nonsense mutations is used. Insertions or deletions of at least one base pair in a gene cause frame shift mutations, the result of which is that false amino acids are incorporated or translation is terminated prematurely. Deletions of several codons typically lead to a complete loss of enzyme activity. Instructions for the production of such mutations form part of the state of the art and can be found in well-known textbooks on genetics and molecular biology, e.g. the textbook by Knippers ("Molekulare Genetik" ("Molecular Genetics"), 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), the textbook by Winnacker ("Gene und Klone" ("From Genes to Clones"), VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or the textbook by Hagemann ("Allgemeine Genetik" ("General Genetics"), Gustav Fischer Verlag, Stuttgart, 1986).

An example of a plasmid by means of which the pckA gene of Escherichia coli can be attenuated and, in particular, switched off by position-specific mutagenesis is plasmid pMAK705ApckA (Figure 1). It contains only part of the 5' region and part of the 3' region of the pckA gene. A 349 bp segment of the coding region is missing (deletion). The sequence of this DNA, which can be used for mutagenesis of the pckA gene, is represented in SEQ ID No. 3.

The deletion mutation of the pckA gene can be incorporated into suitable strains by gene or allele exchange.

A common method is the method of gene exchange using a conditionally replicating pSC101 derivative, pMAK705, as

described by Hamilton et al. (Journal of Bacteriology 174, 4617-4622 (1989)). Other methods described in the state of the art, for example that of Martinez-Morales et al. (Journal of Bacteriology, 7143-7148 (1999)) or that of Boyd et al. (Journal of Bacteriology 182, 842-847 (2000)), can also be used.

When exchange has been carried out, the form of the $\Delta pckA$ allele represented in SEQ ID No. 4, which is a further subject of the invention, is present in the strain in question.

Mutations in the *pckA* gene or mutations involving expression of the *pckA* gene can also be transferred to different strains by conjugation or transduction.

Furthermore, for the production of L-amino acids, especially L-threonine, with strains of the family Enterobacteriaceae, it can be advantageous not only to attenuate the *pckA* gene but also to amplify one or more enzymes of the known threonine biosynthetic pathway, or enzymes of the anaplerotic metabolism, or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate.

In this context the term "amplification" describes the increase in the intracellular activity, in a microorganism, of one or more enzymes or proteins which are coded for by the appropriate DNA, for example by increasing the copy number of the gene(s), using a strong promoter or using a gene coding for an appropriate enzyme or protein with a high activity, and optionally combining these measures.

By amplification measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or

the activity or concentration of the protein in the starting microorganism.

Thus, for example, one or more genes selected from the group comprising:

- 5 • the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene coding for pyruvate carboxylase (DE-A-19 831 609),
- 10 • the pps gene coding for phosphoenolpyruvate synthase (Molecular and General Genetics 231, 332 (1992)),
- the ppc gene coding for phosphoenolpyruvate carboxylase (Gene 31, 279-283 (1984)),
- the pntA and pntB genes coding for transhydrogenase
15 (European Journal of Biochemistry 158, 647-653 (1986)),
- the rhtB gene for homoserine resistance (EP-A-0994190), and
- the rhtC gene for threonine resistance (EP-A-1013765),
- the gdhA gene coding for glutamate dehydrogenase (Gene
20 27:193-199 (1984))

can be simultaneously amplified and, in particular, overexpressed.

Furthermore, for the production of L-amino acids, especially L-threonine, it can be advantageous not only to
25 attenuate the pckA gene but also to attenuate and, in particular, switch off one or more genes selected from the group comprising:

- the tdh gene coding for threonine dehydrogenase
(Ravnikar and Somerville, Journal of Bacteriology 169,
4716-4721 (1987)),
- the mdh gene coding for malate dehydrogenase
5 (EC 1.1.1.37) (Vogel et al., Archives in Microbiology
149, 36-42 (1987)),
- the gene product of the open reading frame (orf) yjfA
(Accession Number AAC77180 of the National Center for
Biotechnology Information (NCBI, Bethesda, MD, USA) and
10 SEQ ID No. 5), and
- the gene-product of the open reading frame (orf) ytfP
(Accession Number AAC77179 of the National Center for
Biotechnology Information (NCBI, Bethesda, MD, USA) and
SEQ ID No. 5),

15 or to reduce the expression.

It is preferred to attenuate the open reading frame yjfA
and/or the open reading frame ytfP.

It is also possible according to the invention to attenuate
the open reading frames yjfA and/or ytfP independently of
20 the pckA gene, in order to achieve an improvement in the
amino acids, in particular L-threonine production.

The invention accordingly also provides a process,
characterized in that the following steps are carried out:

- d) fermentation of microorganisms of the
25 Enterobacteriaceae family in which at least the
open reading frame yjfA and/or ytfP is attenuated,
- e) enrichment of the L-amino acid in the medium or in
the cells of the microorganisms of the
Enterobacteriaceae family, and

- f) isolation of the L-threonine, constituents of the fermentation broth and the biomass in its entirety or portions thereof optionally being isolated as a solid product together with the L-amino acid.
- 5 An example of a plasmid by means of which the open reading frames yjfa and ytfP of Escherichia coli can be attenuated and, in particular, switched off by position-specific mutagenesis is plasmid pMAK705Δyjfa (Figure 2). It contains only the 5' and 3' flanks of the ytfP-yjfa region, including very short residues of the open reading frames yjfa and ytfP. A 337 bp long part of the ytfP-yjfa region is missing (deletion). The sequence of this DNA, which can be used for mutagenesis of the ytfP-yjfa region, is represented in SEQ ID No. 6.
- 10
- 15 An further example of a plasmid by means of which the open reading frames yjfa and ytfP of Escherichia coli can be attenuated and, in particular, switched off by position-specific mutagenesis is the plasmid pMAK705Δ90bp (Figure 5). It also contains only the 5' and 3' flanks of the ytfP-yjfa region including very short residues of the open reading frames yjfa and ytfP. A 90 bp long part of the ytfP-yjfa region is missing (deletion). The sequence of this DNA, which can be used for mutagenesis of the ytfP-yjfa region, is represented in SEQ ID No. 7.
- 20
- 25 This deletion mutation can be incorporated into suitable strains by gene or allele replacement. It is also possible to transfer mutations in the open reading frames yjfa and/or ytfP or mutations affecting expression of these open reading frames into various strains by conjugation or transduction.
- 30

When replacement has been carried out, the form of the ΔytfP and Δyjfa allele represented in SEQ ID No. 6 or SEQ

ID No. 7, which are a further subject of the invention, is present in the strain in question.

Furthermore, for the production of L-amino acids, especially L-threonine, it can be advantageous, in addition
5 to the individual or joint attenuation of the *pckA* gene or of the open reading frames *yjfa* and/or *ytfP*, to switch off undesired secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.),
10 Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can be cultivated by the batch process or the fed batch process. A summary of known cultivation methods is provided in the textbook by Chmiel (Bioprozesstechnik 1.
15 Einführung in die Bioverfahrenstechnik (Bioprocess Technology 1. Introduction to Bioengineering) (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Bioreactors and Peripheral Equipment) (Vieweg Verlag,
20 Brunswick/Wiesbaden, 1994)).

The culture medium to be used must appropriately meet the demands of the particular strains. Descriptions of culture media for various microorganisms can be found in the
25 handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington DC, USA, 1981).

Carbon sources which can be used are sugars and carbohydrates, e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils
30 and fats, e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, e.g. palmitic acid, stearic acid and linoleic acid, alcohols, e.g. glycerol and ethanol, and

organic acids, e.g. acetic acid. These substances can be used individually or as a mixture.

Nitrogen sources which can be used are organic nitrogen-containing compounds such as peptones, yeast extract, meat
5 extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used individually or as a mixture.

10 Phosphorus sources which can be used are phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium salts. The culture medium must also contain metal salts, e.g. magnesium sulfate or iron sulfate, which are necessary for
15 growth. Finally, essential growth-promoting substances such as amino acids and vitamins can be used in addition to the substances mentioned above. Suitable precursors can also be added to the culture medium. Said feed materials can be added to the culture all at once or fed in
20 appropriately during cultivation.

The pH of the culture is controlled by the appropriate use of basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds such as phosphoric acid or sulfuric acid. Foaming can be
25 controlled using antifoams such as fatty acid polyglycol esters. The stability of plasmids can be maintained by adding suitable selectively acting substances, e.g. antibiotics, to the medium. Aerobic conditions are maintained by introducing oxygen or oxygen-containing
30 gaseous mixtures, e.g. air, into the culture. The temperature of the culture is normally 25°C to 45°C and preferably 30°C to 40°C. The culture is continued until the formation of L-amino acids or L-threonine has reached a

maximum. This objective is normally achieved within 10 hours to 160 hours.

L-Amino acids can be analyzed by means of anion exchange chromatography followed by ninhydrin derivation, as

- 5 described by Spackman et al. (Analytical Chemistry 30, 1190 (1958)), or by reversed phase HPLC, as described by Lindroth et al. (Analytical Chemistry 51, 1167-1174 (1979)).

- 10 A pure culture of the Escherichia coli K-12 strain DH5 α /pMAK705 was deposited on 12th September 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 13720.

- 15 A pure culture of the Escherichia coli K-12 strain MG442 Δ pckA was deposited on 2nd October 2000 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the
20 Budapest Treaty as DSM 13761.

- A pure culture of the Escherichia coli K-12 strain B-3996kur Δ t Δ dh Δ pckA/pVIC40 was deposited on 9th March 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell
25 Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 14150.

- A pure culture of the Escherichia coli K-12 strain MG442 Δ 90yjfA was deposited on 9th May 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ =
30 German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty as DSM 14289.

It is also possible according to the invention individually to attenuate the open reading frames ytfP and yjfA in order to improve the production of L-amino acids.

5 The process according to the invention is used for the preparation of L-amino acids, e.g. L-threonine, L-isoleucine, L-methionine, L-homoserine and L-lysine, especially L-threonine, by fermentation.

The present invention is illustrated in greater detail below with the aid of Examples.

10 The isolation of plasmid DNA from Escherichia coli and all the techniques for restriction, Klenow treatment and alkaline phosphatase treatment were carried out as described by Sambrook et al. (Molecular cloning - A laboratory manual (1989), Cold Spring Harbor Laboratory Press).
15 Unless indicated otherwise, the transformation of Escherichia coli was carried out as described by Chung et al. (Proceedings of the National Academy of Sciences USA 86, 2172-2175 (1989)).

The incubation temperature for the preparation of strains and transformants was 37°C. Temperatures of 30°C and 44°C were used in the gene exchange process of Hamilton et al.

Example 1

Construction of the deletion mutation of the pckA gene

Parts of the 5' and 3' regions of the pckA gene of
25 Escherichia coli K12 were amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. The nucleotide sequence of the pckA gene in E. coli K12 MG1655 (SEQ ID No. 1) was used to synthesize the following PCR primers (MWG Biotech, Ebersberg, Germany):

30 pckA'5'-1: 5' - GATCCGAGCCTGACAGGTTA - 3'
pckA'5'-2: 5' - GCATGCGCTCGGTCAGGTTA - 3'

pckA'3'-1: 5' - AGGCCTGAAGATGGCACTATCG - 3'

pckA'3'-2: 5' - CCGGAGAAGCGTAGGTGTTA - 3'.

The chromosomal E. coli K12 MG1655 DNA used for the PCR was isolated with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. An approx. 500 bp DNA fragment from the 5' region of the pckA gene (denoted as pck1) and an approx. 600 bp DNA fragment from the 3' region of the pckA gene (denoted as pck2) could be amplified with the specific primers under standard PCR conditions (Innis et al. (1990), PCR Protocols. A Guide to Methods and Applications, Academic Press) using Taq DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR products were each ligated with vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions and transformed into E. coli strain TOP10F'. Plasmid-carrying cells were selected on LB agar containing 50 µg/ml of ampicillin. After isolation of the plasmid DNA, vector pCR2.1TOPOpck2 was cleaved with the restriction enzymes StuI and XbaI and, after separation in 0.8% agarose gel, the pck2 fragment was isolated with the aid of the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After isolation of the plasmid DNA, vector pCR2.1TOPOpck1 was cleaved with the enzymes EcoRV and XbaI and ligated to the isolated pck2 fragment. The E. coli strain DH5α was transformed with the ligation mixture and plasmid-carrying cells were selected on LB agar containing 50 µg/ml of ampicillin. After isolation of the plasmid DNA, control cleavage with the enzymes SpeI and XbaI was used to detect plasmids containing, in cloned form, the mutagenic DNA sequence represented in SEQ ID No. 3. One of the plasmids was denoted as pCR2.1TOPOΔpckA.

Example 2Construction of exchange vector pMAK705 Δ pckA

After restriction with the enzymes SpeI and XbaI and separation in 0.8% agarose gel, the pckA allele described in Example 1 was isolated from vector pCR2.1TOPO Δ pckA and ligated to plasmid pMAK705 (Hamilton et al., Journal of Bacteriology 174, 4617-4622 (1989)) which had been digested with the enzyme XbaI. DH5 α was transformed with the ligation mixture and plasmid-carrying cells were selected on LB agar containing 20 μ g/ml of chloramphenicol. After isolation of the plasmid DNA and cleavage with the enzymes HpaI, KpnI, HindIII, SalI and PstI, successful cloning was detected. The exchange vector formed, pMAK705 Δ pckA (= pMAK705 Δ pckA), is shown in Figure 1.

15 Example 3

Position-specific mutagenesis of the pckA gene in the E. coli strain MG442

The L-threonine-producing E. coli strain MG442 is described in patent US-A-4,278,765 and deposited in the Russian National Collection of Industrial Microorganisms (VKPM, Moscow, Russia) as CMIM B-1628.

The strain MG442 has a resistance to α -amino- β -hydroxyvaleric acid and has an optionally partial and compensable need for L-isoleucine.

25 For exchange of the chromosomal pckA gene for the plasmid-coded deletion construct, MG442 was transformed with plasmid pMAK705 Δ pckA. The gene exchange was carried out by the selection method described by Hamilton et al. (Journal of Bacteriology 174, 4617-4622 (1989)) and was verified by
30 standard PCR methods (Innis et al. (1990), PCR Protocols. A

Guide to Methods and Applications, Academic Press) using the following oligonucleotide primers:

pckA'5'-1: 5' - GATCCGAGCCTGACAGGTTA - 3'

pckA'3'-2: 5' - CCGGAGAAGCGTAGGTGTTA - 3'

- 5 The strain obtained was denoted as MG442ΔpckA.

Example 4

Preparation of L-threonine with the strain MG442ΔpckA

- MG442ΔpckA was cultivated on minimum medium of the following composition: 3.5 g/l of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 g/l of KH_2PO_4 , 1 g/l of NH_4Cl , 0.1 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/l of glucose and 20 g/l of agar. The formation of L-threonine was checked in 10 ml batch cultures contained in 100 ml Erlenmeyer flasks. These were inoculated with 10 ml of a preculture medium of the following composition: 2 g/l of yeast extract, 10 g/l of $(\text{NH}_4)_2\text{SO}_4$, 1 g/l of KH_2PO_4 , 0.5 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g/l of CaCO_3 and 20 g/l of glucose, and incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). 250 μl of this preculture were transferred to 10 ml of a production medium (25 g/l of $(\text{NH}_4)_2\text{SO}_4$, 2 g/l of KH_2PO_4 , 1 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/l of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g/l of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 30 g/l of CaCO_3 , 20 g/l of glucose) and incubated for 48 hours at 37°C. After incubation, the optical density (OD) of the culture suspension was determined with an LP2W photometer from Dr. Lange (Berlin, Germany) at a measurement wavelength of 660 nm.

- The concentration of L-threonine formed was then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by means of ion exchange chromatography and postcolumn reaction with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	6.0	1.5
MG442 Δ pckA	5.4	3.7

Example 5

- 5 Preparation of L-threonine with the strain
MG442 Δ pckA/pMW218gdhA

5.1 Amplification and cloning of the *gdhA* gene

The glutamate dehydrogenase gene from *Escherichia coli* K12 is amplified using the polymerase chain reaction (PCR) and
10 synthetic oligonucleotides. Starting from the nucleotide
sequence for the *gdhA* gene in *E. coli* K12 MG1655 (gene
library: Accession No. AE000270 and No. AE000271) PCR
primers are synthesized (MWG Biotech, Ebersberg, Germany):

- Gdh1: 5' - TGAACACTTCTGGCGGTACG - 3'
15 Gdh2: 5' - CCTCGGCGAAGCTAATATGG - 3'

The chromosomal *E. coli* K12 MG1655 DNA employed for the PCR is isolated according to the manufacturers instructions with "QIAGEN Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 2150 bp in size, which comprises the
20 *gdhA* coding region and approx. 350 bp 5'-flanking and
approx. 450 bp 3'-flanking sequences, can be amplified with
the specific primers under standard PCR conditions (Innis
et al.: PCR Protocols. A Guide to Methods and Applications,
1990, Academic Press) with the Pfu-DNA polymerase (Promega
25 Corporation, Madison, USA). The PCR product is cloned in

the plasmid pCR2.1TOPO and transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands, Product Description TOPO TA Cloning Kit, Cat. No. K4500-01).

Successful cloning is demonstrated by cleavage of the
5 plasmid pCR2.1TOPOgdhA with the restriction enzymes EcoRI and EcoRV. For this, the plasmid DNA is isolated by means of the "QIAprep Spin Plasmid Kits" (QIAGEN, Hilden, Germany) and, after cleavage, separated in a 0.8 % agarose gel.

10 5.2 Cloning of the gdhA gene in the plasmid vector pMW218

The plasmid pCR2.1TOPOgdhA is cleaved with the enzyme EcoRI, the cleavage batch is separated on 0.8% agarose gel and the gdhA fragment 2.1 kbp in size is isolated with the aid of the "QIAquick Gel Extraction Kit" (QIAGEN, Hilden,
15 Germany). The plasmid pMW218 (Nippon Gene, Toyama, Japan) is cleaved with the enzyme EcoRI and ligated with the gdhA fragment. The E. coli strain DH5 α is transformed with the ligation batch and pMW218-carrying cells are selected by plating out on LB agar (Lennox, Virology 1955, 1: 190), to
20 which 20 μ g/ml kanamycin are added.

Successful cloning of the gdhA gene can be demonstrated after plasmid DNA isolation and control cleavage with EcoRI and EcoRV. The plasmid is called pMW218gdhA (Figure 3).

5.3 Preparation of the strain MG442 Δ pckA/pMW218gdhA

25 The strain MG442 Δ pckA obtained in Example 3 and the strain MG442 are transformed with the plasmid pMW218gdhA and transformants are selected on LB agar, which is supplemented with 20 μ g/ml kanamycin. The strains MG442 Δ pckA/pMW218gdhA and MG442/pMW218gdhA are formed in
30 this manner.

5.4 Preparation of L-threonine

The preparation of L-threonine by the strains MG442 Δ pckA/pMW218gdhA and MG442/pMW218gdhA is tested as described in Example 4. The minimal medium and the
5 preculture medium are additionally supplemented with 20 μ g/ml kanamycin.

The result of the experiment is summarized in Table 2.

Table 2

Strain	OD (660 nm)	L-Threonine g/l
MG442	6.0	1.5
MG442 Δ pckA	5.4	3.7
MG442/pMW218gdhA	5.6	2.6
MG442 Δ pckA/pMW218gdhA	5.5	4.0

10 Example 6

Preparation of L-threonine with the strain MG442 Δ pckA/pMW219rhtC

6.1 Amplification of the rhtC gene

The rhtC gene from Escherichia coli K12 is amplified using
15 the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence for the rhtC gene in E. coli K12 MG1655 (gene library: Accession No. AE000458, Zakataeva et al. (FEBS Letters 452, 228-232 (1999)), PCR primers are synthesized (MWG Biotech,
20 Ebersberg, Germany):

RhtC1: 5' - CTGTTAGCATCGGCGAGGCA - 3'

RhtC2: 5' - GCATGTTGATGGCGATGACG - 3'

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturers instructions
5 with "QIAGEN Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 800 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al.: PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) with Pfu-DNA polymerase (Promega
10 Corporation, Madison, USA).

6.2 Cloning of the rhtC gene in the plasmid vector pMW219

The plasmid pMW219 (Nippon Gene, Toyama, Japan) is cleaved with the enzyme SamI and ligated with the rhtC-PCR
15 fragment. The E. coli strain DH5 α is transformed with the ligation batch and pMW219-carrying cells are selected on LB agar, which is supplemented with 20 μ g/ml kanamycin. Successful cloning can be demonstrated after plasmid DNA isolation and control cleavage with KpnI, HindIII and NcoI. The plasmid pMW219rhtC is shown in Figure 4.

20 6.3 Preparation of the strain MG442 Δ pckA/pMW219rhtC

The strain MG442 Δ pckA obtained in Example 3 and the strain MG442 are transformed with the plasmid pMW219rhtC and transformants are selected on LB agar, which is
supplemented with 20 μ g/ml kanamycin. The strains
25 MG442 Δ pckA/pMW219rhtC and MG442/pMW219rhtC are formed in this manner.

6.4 Preparation of L-threonine

The preparation of L-threonine by the strains MG442 Δ pckA/pMW219rhtC and MG442/pMW219rhtC is tested as
30 described in Example 4. The minimal medium and the

preculture medium are additionally supplemented with 20 µg/ml kanamycin.

The result of the experiment is summarized in Table 3.

Table 3

Strain	OD (660 nm)	L-Threonine g/l
MG442	6.0	1.5
MG442ΔpckA	5.4	3.7
MG442/pMW219rhtC	5.2	2.9
MG442ΔpckA/pMW219rhtC	4.8	4.4

5

Example 7

Preparation of L-threonine with the strain
B-3996kurAtdhΔpckA/pVIC40

10 The L-threonine-producing E. coli strain B-3996 is described in US-A- 5,175,107 and deposited at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

15 The strain B-3996 has, inter alia, a resistance to α-amino-β-hydroxyvaleric acid, has an attenuated, in particular switched-off, or defective threonine dehydrogenase, has an enhanced homoserine dehydrogenase I aspartate kinase I in the feed back resistant form, has an optionally partial and compensable need for L-isoleucine and has the ability to utilize sucrose.

7.1 Preparation of the strain B-3996kur Δ tdh Δ pckA/pVIC40

After culture in antibiotic-free complete medium for approximately ten generations, a derivative of strain B-3996 which no longer contains the plasmid pVIC40 is
5 isolated. The strain formed is streptomycin-sensitive and is designated B-3996kur.

The method described by Hamilton et al. (Journal of Bacteriology (1989) 171: 4617-4622), which is based on the use of the plasmid pMAK705 with a temperature-sensitive
10 replicon, was used for incorporation of a deletion into the tdh gene. The plasmid pDR121 (Ravnikar and Somerville, Journal of Bacteriology (1987) 169:4716-4721) contains a DNA fragment from E. coli 3.7 kilo-base pairs (kbp) in size, on which the tdh gene is coded. To generate a
15 deletion of the tdh gene region, pDR121 is cleaved with the restriction enzymes ClaI and EcoRV and the DNA fragment 5 kbp in size isolated is ligated, after treatment with Klenow enzyme. The ligation batch is transformed in the E. coli strain DH5 α and plasmid-carrying cells are selected on
20 LB agar, to which 50 μ g/ml ampicillin are added.

Successful deletion of the tdh gene can be demonstrated after plasmid DNA isolation and control cleavage with EcoRI. The EcoRI fragment 1.7 kbp in size is isolated, and ligated with the plasmid pMAK705, which is partly digested
25 with EcoRI. The ligation batch is transformed in DH5 α and plasmid-carrying cells are selected on LB agar, to which 20 μ g/ml chloramphenicol are added. Successful cloning is demonstrated after isolation of the plasmid DNA and cleavage with EcoRI. The pMAK705 derivative formed is
30 designated pDM32.

For the gene replacement, B-3996kur is transformed with the plasmid pDM32. The replacement of the chromosomal tdh gene with the plasmid-coded deletion construct is carried out by

the selection process described by Hamilton et al. and is verified by standard PCR methods (Innis et al. (1990), PCR Protocols. A Guide to Methods and Applications, Academic Press) with the following oligonucleotide primers:

- 5 Tdh1: 5'-TCGCGACCTATAAGTTTGGG-3'
Tdh2: 5'-AATACCAGCCCTTGTTTCGTG-3'.

The strain formed is tested for kanamycin sensitivity and is designated B-3996kur Δ tdh.

- 10 For the position-specific mutagenesis of the pckA gene, B-3996kur Δ tdh is transformed with the replacement vector pMAK705 Δ pckA described in Example 2. The replacement of the chromosomal pckA gene by the plasmid-coded deletion construct is carried out as described in Example 3. The strain obtained is called B-3996kur Δ tdh Δ pckA.

- 15 B-3996kur Δ tdh and B-3996kur Δ tdh Δ pckA are transformed with the plasmid pVIC40 isolated from B-3996 and plasmid-carrying cells are selected on LB agar with 20 μ g/ml streptomycin. In each case a selected individual colony is called B-3996kur Δ tdh/pVIC40 and B-3996kur Δ tdh Δ pckA/pVIC40.

20 7.2 Preparation of L-threonine

- The preparation of L-threonine by the strains B-3996kur Δ tdh/pVIC40 and B-3996kur Δ tdh Δ pckA/pVIC40 is tested as described in Example 4. The minimal medium, the preculture medium and the production medium are
25 additionally supplemented with 20 μ g/ml streptomycin.

The result of the experiment is summarized in Table 4.

Table 4

Strain	OD (660 nm)	L-Threonine g/l
B-3996kur Δ tdh/pVIC40	4.7	6.26
B-3996kur Δ tdh Δ pckA/pVIC40	4.9	8.92

Example 8

Preparation of L-lysine with the strain TOC21R Δ pckA

- 5 The L-lysine-producing *E. coli* strain pDA1/TOC21R is described in the patent application F-A-2511032 and deposited at the Collection Nationale de Culture de Microorganisme (CNCM = National Microorganism Culture Collection, Pasteur Institute, Paris, France) under number
10 I-167. The strain and the plasmid-free host are also described by Dauce-Le Reverend et al. (European Journal of Applied Microbiology and Biotechnology 15:227-231 (1982)) under the name TOC21/pDA1.

- 8.1 Position-specific mutagenesis of the pckA gene in the
15 *E. coli* strain TOC21R

After culture in antibiotic-free LB medium for approximately six generations, a derivative of strain pDA1/TOC21R which no longer contains the plasmid pDA1 is isolated. The strain formed is tetracycline-sensitive and
20 is called TOC21R.

For replacement of the chromosomal pckA gene by the plasmid-coded deletion construct, TOC21R is transformed with the plasmid pMAK705 Δ pckA (Example 2). The gene replacement is carried out by the selection method
25 described by Hamilton et al. (1989) Journal of Bacteriology

174, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with the following oligonucleotide primers:

- 5 pckA'5'-1: 5' - GATCCGAGCCTGACAGGTTA - 3'
pckA'3'-2: 5' - CCGGAGAAGCGTAGGTGTTA - 3'

The strain obtained is called TOC21RΔpckA.

8.2 Preparation of L-lysine with the strain TOC21RΔpckA

- The formation of L-lysine by the strains TOC21RA pckA and
10 TOC21R is checked in batch cultures of 10 ml contained in
100 ml conical flasks. For this, 10 ml of preculture medium
of the following composition: 2 g/l yeast extract, 10 g/l
(NH₄)₂SO₄, 1 g/l KH₂PO₄, 0.5 g/l MgSO₄*7H₂O, 15 g/l CaCO₃, 20
g/l glucose are inoculated and the batch is incubated for
15 16 hours at 37°C and 180 rpm on an ESR incubator from
Kühner AG (Birsfelden, Switzerland). 250 µl of this
preculture are transinoculated into 10 ml of production
medium (25 g/l (NH₄)₂SO₄, 2 g/l KH₂PO₄, 1 g/l MgSO₄*7H₂O,
0.03 g/l FeSO₄*7H₂O, 0.018 g/l MnSO₄*1H₂O, 30 g/l CaCO₃, 20
20 g/l glucose, 25 mg/l L-isoleucine and 5 mg/l thiamine) and
the batch is incubated for 72 hours at 37°C. After the
incubation the optical density (OD) of the culture
suspension is determined with an LP2W photometer from Dr.
Lange (Berlin, Germany) at a measurement wavelength of 660
25 nm.

- The concentration of L-lysine formed is then determined in
the sterile-filtered culture supernatant with an amino acid
analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion
exchange chromatography and post-column reaction with
30 ninhydrin detection.

The result of the experiment is shown in Table 5.

Table 5

Strain	OD (660 nm)	L-Lysine g/l
TOC21R	1.0	1.14
TOC21R Δ pckA	1.0	1.27

Example 9

Preparation of L-isoleucine with the strain

5 B-3996kur Δ tdh Δ lvA⁺ Δ pckA/pVIC40

9.1 Preparation of the strain B-
3996kur Δ tdh Δ lvA⁺ Δ pckA/pVIC40

10 The strain B-3996kur Δ tdh, which is in need of L-isoleucin, obtained in Example 7.1 is transduced with the aid of the phage Plkc (Lennox, Virology 1, 190-206 (1955); Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory 1972) and L-isoleucine-prototrophic transductants are isolated.

15 For this, the phage Plkc is multiplied on the strain MG1655 (Guyer et al., Cold Spring Harbor Symposium of Quantitative Biology 45, 135-140 (1981) and Blattner et al., Science. 277, 1453-1462 (1997)) and the phage lysate is employed for the transduction of the strain B-3996kur Δ tdh. The multiplicity of the infection is approximately 0.2.

20 Selection for L-isoleucine-prototrophic transductants is carried out on minimal agar, which contains 2 g/l glucose and 10 mg/l L-threonine. An L-isoleucine-prototrophic transductant is isolated, smeared on to LB agar for purification or isolation and called B-3996kur Δ tdh Δ lvA⁺.

The pckA gene of the strain B-3996kur Δ tdhilvA⁺ is then replaced, as described in Example 3, by the Δ pckA allele prepared in Example 1 and 2. The strain obtained is called B-3996kur Δ tdhilvA⁺ Δ pckA.

- 5 The strains B-3996kur Δ tdhilvA⁺ and B-3996kur Δ tdhilvA⁺ Δ pckA are transformed with the plasmid pVIC40 isolated from strain B-3996 and plasmid-carrying cells are selected on LB agar, which is supplemented with 20 μ g/ml streptomycin. In each case a selected individual colony is called B-
10 3996kur Δ tdhilvA⁺ Δ pckA/pVIC40 and B-3996kur Δ tdhilvA⁺/pVIC40.

9.2 Preparation of L-isoleucine

- The preparation of L-isoleucine by the strains B-3996kur Δ tdhilvA⁺/pVIC40 and B-
15 3996kur Δ tdhilvA⁺ Δ pckA/pVIC40 is tested under the test conditions as described in Example 4. The minimal medium, the preculture medium and the production medium are additionally supplemented with 20 μ g/ml streptomycin.

The result of the experiment is shown in Table 6.

Table 6

Strain	OD (660 nm)	L-Isoleucine mg/l
B-3996kur Δ tdhilvA ⁺ /pVIC40	5.8	57
B-3996kur Δ tdhilvA ⁺ Δ pckA/pVIC40	5.7	70

Example 10

Preparation of L-valine with the strain B-12288 Δ pckA

The L-valine-producing E. coli strain AJ 11502 is described in the patent specification US-A-4391907 and deposited at
5 the National Center for Agricultural Utilization Research (Peoria, Illinois, USA) as NRRL B-12288.

10.1 Position-specific mutagenesis of the pckA gene in the E. coli strain B-1288

After culture in antibiotic-free LB medium for
10 approximately six generations, a plasmid-free derivative of strain AJ 11502 is isolated. The strain formed is ampicillin-sensitive and is called AJ11502kur.

For replacement of the chromosomal pckA gene by the plasmid-coded deletion construct, AJ11502kur is transformed
15 with the plasmid pMAK705 Δ pckA (see Example 2). The gene replacement is carried out by the selection method described by Hamilton et al. (1989) Journal of Bacteriology 174, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A Guide to Methods and
20 Applications, Academic Press) with the following oligonucleotide primers:

pckA'5'-1: 5' - GATCCGAGCCTGACAGGTTA - 3'

pckA'3'-2: 5' - CCGGAGAAGCGTAGGTGTTA - 3'

The strain obtained is called AJ11502kur Δ pckA. The plasmid
25 described in the patent specification US-A-4391907, which carries the genetic information in respect of valine production, is isolated from strain NRRL B-12288. The strain AJ11502kur Δ pckA is transformed with this plasmid. One of the transformants obtained is called B-12288 Δ pckA.

10.2 Preparation of L-valine with the strain B-12288 Δ pckA

The formation of L-valine by the strains B-12288 Δ pckA and NRRL B-12288 is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of
5 preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(\text{NH}_4)_2\text{SO}_4$, 1 g/l KH_2PO_4 , 0.5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g/l CaCO_3 , 20 g/l glucose and 50 mg/l ampicillin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden,
10 Switzerland). 250 μl of this preculture are transinoculated into 10 ml of production medium (25 g/l $(\text{NH}_4)_2\text{SO}_4$, 2 g/l KH_2PO_4 , 1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.018 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 30 g/l CaCO_3 , 20 g/l glucose, 5 mg/l thiamine and 50 mg/l ampicillin) and the batch is incubated for 72
15 hours at 37°C. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from Dr. Lange (Berlin, Germany) at a measurement wavelength of 660 nm.

The concentration of L-valine formed is then determined in
20 the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 7.

25

Table 7

Strain	OD (660 nm)	L-Valine g/l
NRRL B-12288	5.6	0.93
B-12288 Δ pckA	5.5	1.12

Example 11

Construction of deletion mutations of the ytfP-yjfa gene region

The ytfP-yjfa gene region is amplified from Escherichia coli K12 using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the ytfP-yjfa gene region in E. coli K12 MG1655 (SEQ ID No. 5), the following PCR primers are synthesized (MWG Biotech, Ebersberg, Germany):

10 ytfP-1: 5' - GGCGATGTCGCAACAAGCTG - 3'
ytfP-2: 5' - CTGTTTCATGGCCGCTTGCTG - 3'

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturers instructions with "Qiagen Genomic-tips 100/G" (QIAGEN, Hilden, Germany).
15 A DNA fragment approx. 1300 bp in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with Taq-DNA polymerase (Gibco-BRL, Eggenstein, Germany). The PCR product is
20 ligated with the vector pCR2.1TOPO (TOPO TA Cloning Kit, Invitrogen, Groningen, The Netherlands) in accordance with the manufacturers instructions and transformed into the E. coli strain TOP10F'. Selection of plasmid-carrying cells takes place on LB agar, to which 50 µg/ml ampicillin are
25 added. After isolation of the plasmid DNA, successful cloning of the PCR product is checked with the restriction enzymes EcoRI and NsiI.

To generate a 337 bp deletion in the ytfP-yjfa region, the vector pCR2.1TOPOytfP-yjfa is cleaved with the restriction
30 enzymes NdeI and SspI and the DNA fragment 4.8 kbp in size is ligated, after treatment with Klenow enzyme.

To generate a 90 bp deletion, the vector pCR2.1TOPOytfP-yjfA is cleaved with the enzymes NdeI and SphI and the DNA fragment 5 kbp in size is ligated, after treatment with Klenow enzyme.

- 5 The E. coli strain DH5 α is transformed with the ligation batches and plasmid-carrying cells are selected on LB agar, to which 50 μ g/ml ampicillin is added. After isolation of the plasmid DNA those plasmids in which the mutagenic DNA sequence shown in SEQ ID No. 6 and SEQ ID No. 7 is cloned
- 10 are detected by control cleavage with the enzyme EcoRI. The plasmids are called pCR2.1TOPO Δ yjfA and pCR2.1TOPO Δ 90bp.

Example 12

Construction of the replacement vectors pMAK705ΔyjfA and pMAK705Δ90bp

- The ytfP-yjfA alleles described in Example 11 are isolated from the vectors pCR2.1TOPOΔyjfA and pCR2.1TOPOΔ90bp after restriction with the enzymes SacI and XbaI and separation in 0.8% agarose gel, and ligated with the plasmid pMAK705 (Hamilton et al. (1989) Journal of Bacteriology 174, 4617 - 4622), which is digested with the enzymes SacI and XbaI. The ligation batches are transformed in DH5α and plasmid-carrying cells are selected on LB agar, to which 20 μg/ml chloramphenicol are added. Successful cloning is demonstrated after isolation of the plasmid DNA and cleavage with the enzymes SacI and XbaI. The replacement vectors formed, pMAK705ΔyjfA (= pMAK705deltayjfA) and pMAK705Δ90bp (= pMAK705delta90bp), are shown in Figure 2 and in Figure 5.

Example 13

- Position-specific mutagenesis of the ytfP-yjfA gene region in the E. coli strain MG442

- For replacement of the chromosomal ytfP-yjfA gene region with the plasmid-coded 90 bp deletion construct, MG442 is transformed with the plasmid pMAK705Δ90bp. The gene replacement is carried out by the selection method described by Hamilton et al. (1989) Journal of Bacteriology 174, 4617 - 4622) and is verified by standard PCR methods (Innis et al. (1990) PCR Protocols. A Guide to Methods and Applications, Academic Press) with the following oligonucleotide primers:

- ytfP-1: 5' - GGCGATGTCGCAACAAGCTG - 3'
ytfP-2: 5' - CTGTTTCATGCCGCTTGCTG - 3'

The strain obtained is called MG442 Δ 90yjfA.

Example 14

Preparation of L-threonine with the strain MG442 Δ 90yjfA

5 The preparation of L-threonine by the strain MG442 Δ 90yjfA is tested as described in Example 4. The result of the experiment is summarized in Table 8.

Table 8

Strain	OD (660 nm)	L-Threonine g/l
MG442	6.0	1.5
MG442 Δ 90yjfA	5.7	2.1

Example 15

10 Preparation of L-threonine with the strain MG442 Δ 90yjfA Δ pckA

15.1 Preparation of the strain MG442 Δ 90yjfA Δ pckA

15 The pckA gene of the strain MG442 Δ 90yjfA is replaced, as described in Example 3, by the Δ pckA allele (see Example 1 and 2). The strain obtained is called MG442 Δ 90yjfA Δ pckA.

15.2 Preparation of L-threonine

The preparation of L-threonine with the strain MG442 Δ 90yjfA Δ pckA is carried out as described in Example 4. The result is shown in Table 9.

Table 9

Strain	OD (660 nm)	L-Threonine g/l
MG442 Δ 90yjfA	5.7	2.1
MG442 Δ 90yjfA Δ pckA	5.3	3.9

Brief Description of the Figures:

- Figure 1: pMAK705 Δ pckA (= pMAK705deltapckA)
- 5 • Figure 2: pMAK705 Δ yjfA (= pMAK705deltayjfA)
- Figure 3: pMW218gdhA
- Figure 4: pMW219rhtC
- Figure 5: pMAK705 Δ 90bp (= pMAK705delta90bp)

10 The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- cat: Chloramphenicol resistance gene
- rep-ts: Temperature-sensitive replication region of the plasmid pSC101
- 15 • pck1: Part of the 5' region of the pckA gene
- pck2: Part of the 3' region of the pckA gene
- ytfP'-yjfA': DNA sequence containing truncated coding regions of ytfP and yjfA
- kan: Kanamycin resistance gene
- 20 • gdhA: Glutamate dehydrogenase gene

- rhtC: Threonine resistance-imparting gene

The abbreviations for the restriction enzymes have the following meaning

- 5 • BamHI: restriction endonuclease from *Bacillus amyloliquefaciens*
- BglII: restriction endonuclease from *Bacillus globigii*
- ClaI: restriction endonuclease from *Caryophanon latum*
- EcoRI: restriction endonuclease from *Escherichia coli*
- 10 • EcoRV: restriction endonuclease from *Escherichia coli*
- HindIII: restriction endonuclease from *Haemophilus influenzae*
- KpnI: restriction endonuclease from *Klebsiella pneumoniae*
- 15 • PstI: restriction endonuclease from *Providencia stuartii*
- PvuI: restriction endonuclease from *Proteus vulgaris*
- SacI: restriction endonuclease from *Streptomyces achromogenes*
- 20 • SalI: restriction endonuclease from *Streptomyces albus*
- SmaI: restriction endonuclease from *Serratia marcescens*
- 25 • XbaI: restriction endonuclease from *Xanthomonas badrii*

- XhoI: restriction endonuclease from *Xanthomonas*
holcicola

What is claimed is:

1. Fermentation process for the preparation of L-amino acids, especially L-threonine, wherein the following steps are carried out:
 - 5 a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which microorganisms at least the pckA gene or nucleotide sequences coding therefor are attenuated and, in particular, switched off,
 - 10 b) enrichment of the L-amino acid in the medium or in the bacterial cells, and
 - c) isolation of the L-amino acid, constituents of the fermentation broth and the biomass in its entirety or portions thereof optionally being isolated as a
15 solid product together with the L-amino acid.
2. Process according to claim 1, wherein microorganisms are used in which other genes of the biosynthetic pathway of the desired L-amino acid are additionally amplified.
- 20 3. Process according to claim 1, wherein microorganisms are used in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partially switched off.
- 25 4. Process according to claim 1, wherein the expression of the polynucleotide(s) coding for the pckA gene is attenuated and, in particular, switched off.
5. Process according to claim 1, wherein the regulatory and/or catalytic properties of the polypeptide (enzyme protein) coded for by the polynucleotide pckA are
30 reduced.

6. Process according to claim 1, wherein microorganisms of the family Enterobacteriaceae in which one or more genes selected from the group comprising:

- 5 6.1 the thrABC operon coding for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase,
- 6.2 the pyc gene coding for pyruvate carboxylase,
- 6.3 the pps gene coding for phosphoenolpyruvate synthase,
- 10 6.4 the ppc gene coding for phosphoenolpyruvate carboxylase,
- 6.5 the pntA and pntB genes coding for transhydrogenase,
- 6.6 the rhtB gene for homoserine resistance, and
- 15 6.7 the rhtC gene for threonine resistance,
- 6.8 the gdhA gene coding for glutamate dehydrogenase

20 are simultaneously amplified and, in particular, overexpressed are fermented for the preparation of L-amino acids.

7. Process according to claim 1, wherein microorganisms of the family Enterobacteriaceae in which one or more genes selected from the group comprising:

- 25 7.1 the tdh gene coding for threonine dehydrogenase,
- 7.2 the mdh gene coding for malate dehydrogenase,

- 7.3 the gene product of the open reading frame
(orf) yjfA, and
- 7.4 the gene product of the open reading frame
(orf) ytfP;
- 5 are attenuated and, in particular, switched off, or the
expression is reduced, are fermented for the
preparation of L-amino acids.
8. Fermentation process for the preparation of L-amino
acids, especially L-threonine, wherein the following
10 steps are carried out:
- a) fermentation of the microorganisms of the family
Enterobacteriaceae producing the desired L-amino
acid, in which microorganisms at least the open
reading frames yjfA and/or ytfP or nucleotide
15 sequences coding therefor are attenuated and, in
particular, switched off,
- b) enrichment of the L-amino acid in the medium or in
the bacterial cells, and
- c) isolation of the L-amino acid, constituents of the
20 fermentation broth and the biomass in its entirety
or portions thereof optionally being isolated as a
solid product together with the L-amino acid.
9. Process according to claim 1 or 8, wherein L-
isoleucine, L-valine, L-lysine or L-threonine is
25 prepared.
10. L-Amino acid-producing microorganisms of the family
Enterobacteriaceae in which at least the pckA gene or
nucleotide sequences coding therefor are attenuated
and, in particular, switched off.

11. L-Amino acid-producing microorganisms of the family Enterobacteriaceae according to claim 10, which additionally have one or more features selected from the group comprising: a resistance to α -amino- β -hydroxyvaleric acid, an amplified homoserine dehydrogenase I-aspartate kinase I in the feed back resistant form, an optionally compensable partial need for L-isoleucine, an attenuated threonine dehydrogenase and the ability to utilize sucrose.
12. L-Amino acid-producing microorganisms of the family Enterobacteriaceae, in which at least the open reading frame yjfA and/or ytfP or nucleotide sequences coding therefor are attenuated and, in particular, switched off.
13. L-Amino acid-producing microorganisms of the family Enterobacteriaceae according to claim 12, which additionally have one or more features selected from the group comprising: a resistance to α -amino- β -hydroxyvaleric acid, an amplified homoserine dehydrogenase I-aspartate kinase I in the feed back resistant form, an optionally compensable partial need for L-isoleucine, an attenuated threonine dehydrogenase and the ability to utilize sucrose.
14. Plasmid pMAK705 Δ pckA, shown in Figure 1, containing parts of the 5' and 3' regions of the pckA gene, corresponding to SEQ ID No. 3.
15. Plasmid pMAK705 Δ yjfA, shown in Figure 2, containing the 5' and 3' flanks of the ytfP-yjfA region, including very short residues of the open reading frames yjfA- and ytfP, corresponding to SEQ ID No. 6.
16. Plasmid pMAK705 Δ 90bp, shown in Figure 5, containing the 5' and 3' flanks of the ytfP-yjfA region, including very

short residues of the open reading frames yjfA- and ytfP, corresponding to SEQ ID No. 7.

- 5 17. Isolated polynucleotide from microorganisms of the family Enterobacteriaceae containing a polynucleotide sequence coding for the 5' and 3' regions of the pckA gene, shown in SEQ ID No. 4, which is particularly suitable as a constituent of plasmids for the position-specific mutagenesis of the pckA gene.
- 10 18. Isolated polynucleotide from microorganisms of the family Enterobacteriaceae containing the 5' and 3' flanks of the ytfP-yjfA region, shown in SEQ ID No. 6, which is particularly suitable as a constituent of plasmids for the position-specific mutagenesis of the open reading frames ytfP and/or yjfA.
- 15 19. L-Threonine-producing strains of the family Enterobacteriaceae containing a deletion mutation in the pckA gene, corresponding to SEQ ID No. 4.
- 20 20. L-Threonine-producing strains of the family Enterobacteriaceae containing a deletion mutation in the open reading frame ytfP, corresponding to SEQ ID No. 6 or 7.
- 25 21. L-Threonine-producing strains of the family Enterobacteriaceae containing a deletion mutation in the open reading frame yjfA, corresponding to SEQ ID No. 6 or 7.
22. L-Threonine-producing strains of the family Enterobacteriaceae according to claim 19, additionally containing a deletion mutation in the open reading frame ytfP, corresponding to SEQ ID No. 6 or 7.
- 30 23. L-Threonine-producing strains of the family Enterobacteriaceae according to claim 19, additionally

containing a deletion mutation in the open reading frame yjfA, corresponding to SEQ ID No. 6 or 7.

24. L-Threonine-producing strains of the family Enterobacteriaceae according to claims 19, 20 or 21, wherein they have one or more features selected from the group comprising: a resistance to α -amino- β -hydroxyvaleric acid, an amplified homoserine dehydrogenase I-aspartate kinase I in the feed back resistant form, an optionally compensable partial need for L-isoleucine, an attenuated threonine dehydrogenase and the ability to utilize sucrose.
25. *Escherichia coli* K-12 strain MG442 Δ pckA deposited under number DSM 13761 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures).
26. *Escherichia coli* K-12 strain MG442 Δ 90yjfA deposited under number DSM 14289 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures).
27. *Escherichia coli* K-12 strain B3996kur Δ tdhpckA/PVIC40, deposited under number DSM 14150 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures).

Figure 1:

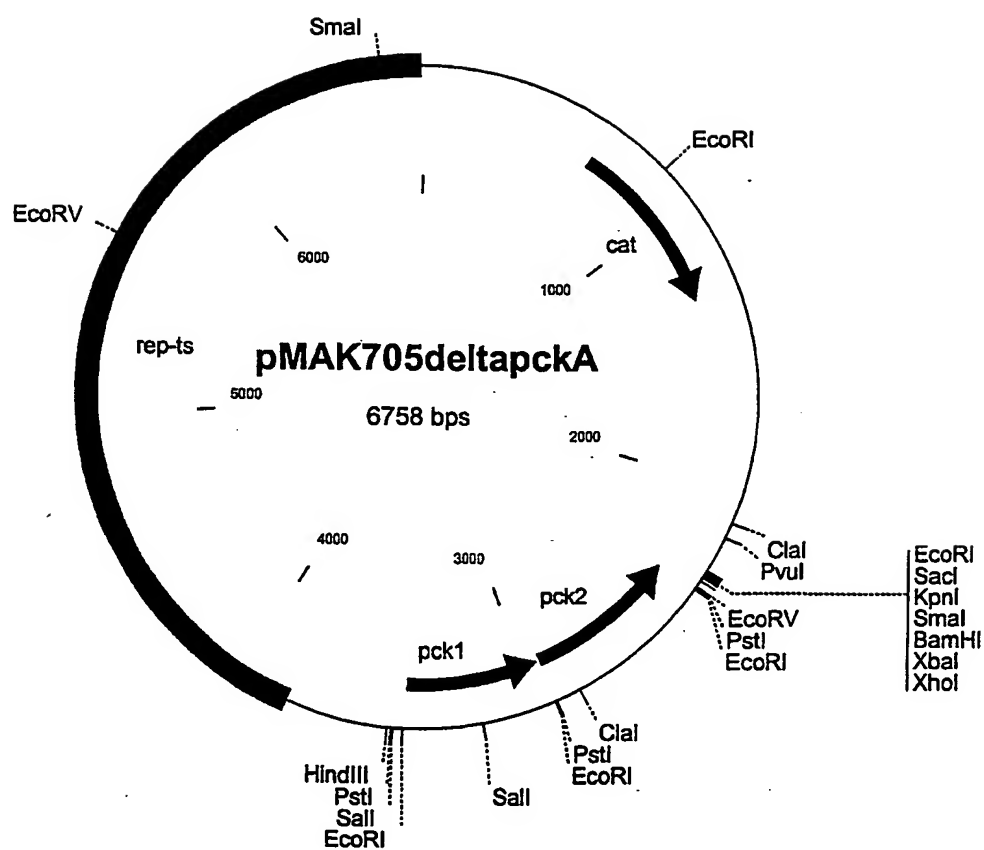


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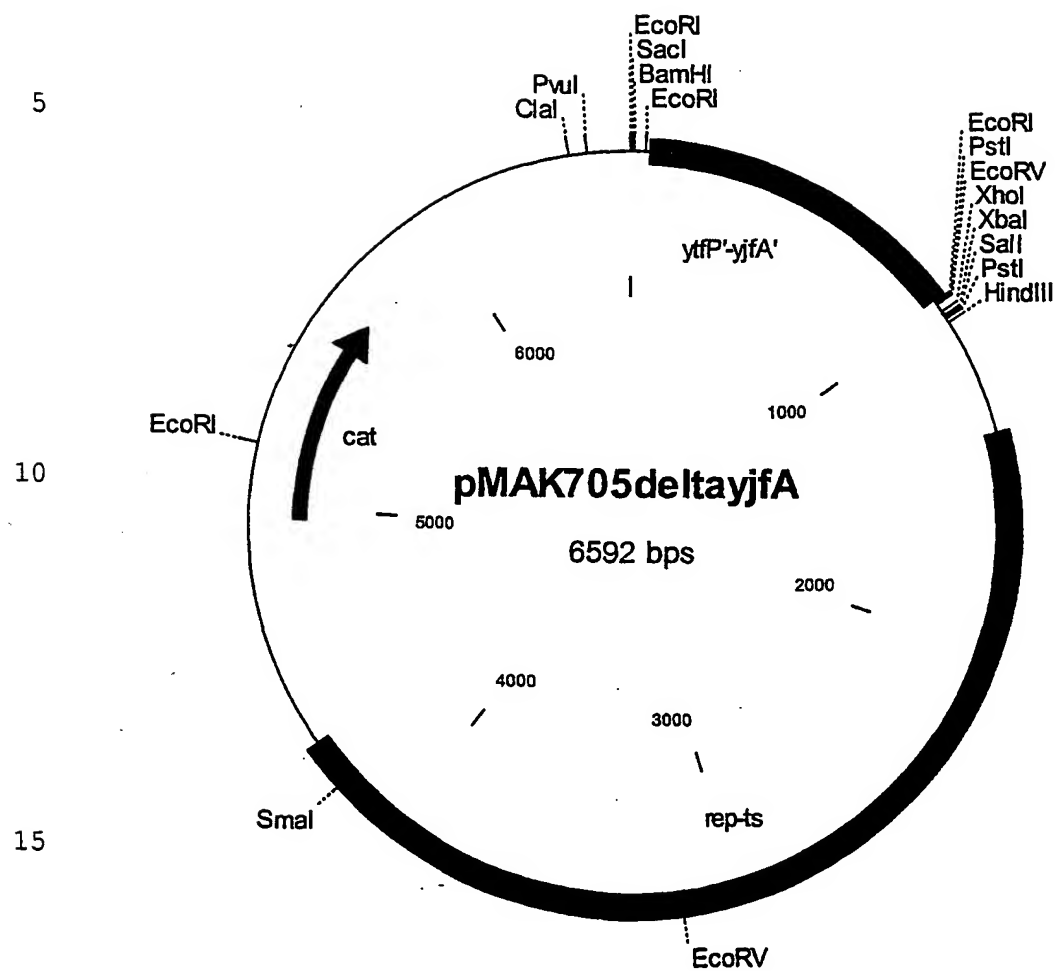


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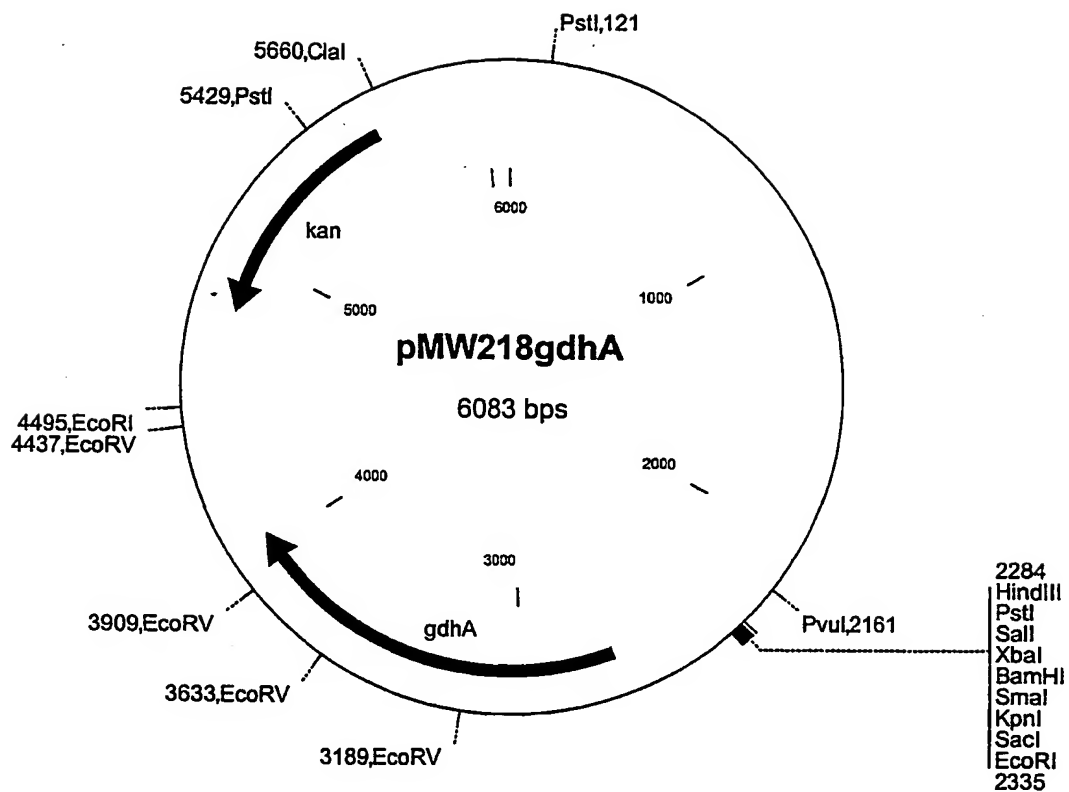


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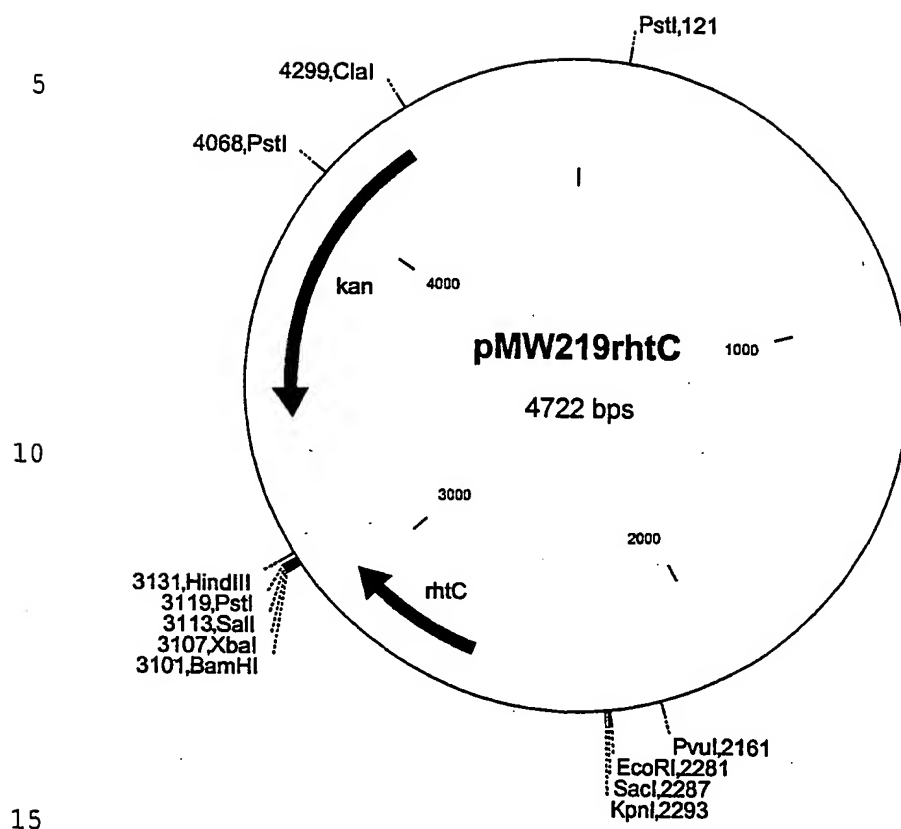
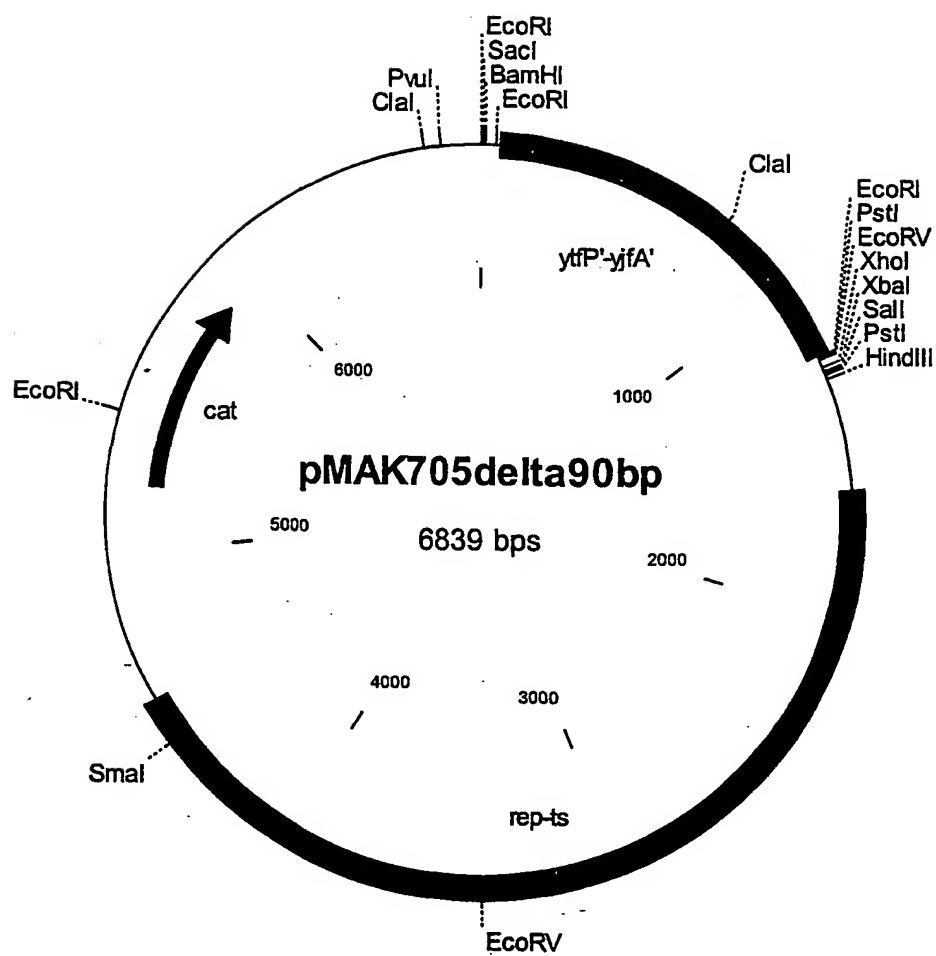


Figure 5:



SEQUENCE PROTOCOL

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    gggagtaggc gactcctccc aggtagtggg cagcggctat gtattgccag gtctgcaagt 240
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20

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GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
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Published:
— with international search report

(88) Date of publication of the international search report:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **FERMENTATION PROCESS FOR THE PREPARATION OF L-AMINO ACIDS USING STRAINS OF THE FAMILY ENTEROBACTERIACEAE**

(57) Abstract: The invention relates to a fermentation process for the preparation of L-amino acids, especially L-threonine, in which the following steps are carried out: a) fermentation of the microorganisms of the family Enterobacteriaceae producing the desired L-amino acid, in which microorganisms at least the *pckA* gene and/or the open reading frames *yjfA* and *ytfP* are, individually or jointly, attenuated and, in particular, switched off, b) enrichment of the L-amino acid in the medium or in the bacterial cells, and c) isolation of the L-amino acid.



WO 02/029080 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/10209

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12P13/04 C12P13/08 C12N1/21 C12N15/11 C12N15/60
 //(C12P13/04,C12R1:19),(C12P13/08,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, SEQUENCE SEARCH, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	EP 1 094 111 A (FORSCHUNGSZENTRUM JÜLICH GMBH) 25 April 2001 (2001-04-25)	1-7, 9-11, 14, 17, 19, 22-25, 27
A	see especially claims 1-11 the whole document	8, 9, 12, 13, 15, 16, 18, 20, 21, 24, 26

	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- *Z* document member of the same patent family

Date of the actual completion of the international search

30 April 2002

Date of mailing of the international search report

17.05.02

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/10209

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1990 GOLDIE H ET AL: "PHYSICAL AND GENETIC ANALYSIS OF THE PHOSPHOENOLPYRUVATE CARBOXYKINASE PCK-A LOCUS FROM ESCHERICHIA-COLI K12" Database accession no. PREV199089092769 XP002193202 abstract & MOLECULAR & GENERAL GENETICS, vol. 220, no. 2, 1990, pages 191-196, ISSN: 0026-8925</p>	10
Y	<p>KRAMER R: "Genetic and physiological approaches for the production of amino acids" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 45, no. 1, 12 February 1996 (1996-02-12), pages 1-21, XP004036833 ISSN: 0168-1656</p>	1-7, 9-11,14, 17,19, 22-25,27
A	<p>see pages 3-5,13 and 15</p>	8,9,12, 13,15, 16,18, 20,21, 24,26
Y	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1990 MEDINA V ET AL: "SEQUENCE OF THE PCK-A GENE OF ESCHERICHIA-COLI K-12 RELEVANCE TO GENETIC AND ALLOSTERIC REGULATION AND HOMOLOGY OF ESCHERICHIA-COLI PHOSPHOENOLPYRUVATE CARBOXYKINASE WITH THE ENZYMES FROM TRYPANOSOMA-BRUCEI AND SACCHAROMYCES-CEREVISIAE" Database accession no. PREV199191038347 XP002193203 cited in the application abstract & JOURNAL OF BACTERIOLOGY, vol. 172, no. 12, 1990, pages 7151-7156, ISSN: 0021-9193</p>	1-7, 9-11,14, 17,19, 22-25,27

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/10209

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; March 1999 (1999-03) PROST JEAN-FRANCOIS ET AL: "Detection of an extended -10 element in the promoter region of the pckA gene encoding phosphoenolpyruvate carboxykinase in Escherichia coli." Database accession no. PREV199900300021 XP002193204 abstract & BIOCHIMIE (PARIS), vol. 81, no. 3, March 1999 (1999-03), pages 197-200, ISSN: 0300-9084</p>	1-7, 9-11,14, 17,19, 22-25,27
A	<p>--- DATABASE CA 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; RIVOLTA, CARLO ET AL: "A 35.7 kb DNA fragment from the Bacillus subtilis chromosome containing a putative 12.3 kb operon involved in hexuronate catabolism and a perfectly symmetrical hypothetical catabolite-responsive element" retrieved from STN Database accession no. 129:63856 XP002197824 abstract & MICROBIOLOGY (READING, U. K.)(1998), 144(4), 877-884 ,</p>	8,9,12, 13,15, 16,18, 20,21, 24,26
A	<p>--- DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1991 BLOMFIELD I C ET AL: "ALLELIC EXCHANGE IN ESCHERICHIA-COLI USING THE BACILLUS-SUBTILIS SAC-B GENE AND A TEMPERATURE-SENSITIVE PSC-101 REPLICON" Database accession no. PREV199192075325 XP002197825 abstract & MOLECULAR MICROBIOLOGY, vol. 5, no. 6, 1991, pages 1447-1458, ISSN: 0950-382X</p>	15,16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 01/10209

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7, 9part., 10, 11, 14, 17, 19, 22, 23, 24part.,
25, 27

Fermentation process for the preparation of L-amino acids using a microorganism of the family Enterobacteriaceae in which at least the pckA gene or nucleotide sequences coding therefore are attenuated; microorganisms useful therefore as well as plasmids and polynucleotides which suppress the pckA gene and PEP carboxykinase, respectively.

2. Claims: 8, 9part., 12, 13, 15, 16, 18, 20, 21, 24part., 26

Fermentation process for the preparation of L-amino acids using a microorganism of the family Enterobacteriaceae in which at least the open reading frames yjfa and/or ytfP or nucleotide sequences coding therefore are attenuated; microorganisms useful therefore as well as plasmids and polynucleotides which suppress the open reading frames yjfa and/or ytfP.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/10209

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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			AU 6410400 A	26-04-2001
			BR 0004957 A	29-05-2001
			CN 1308125 A	15-08-2001
			EP 1094111 A2	25-04-2001
			JP 2001149086 A	05-06-2001
			PL 343339 A1	23-04-2001
